

A freeze-dried diet to test pathogens of Colorado potato beetle

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Abstract

The Colorado potato beetle is an important pest on potato, eggplant, and tomato. Because Colorado potato beetles develop resistance to insecticides quickly, new methods are needed for control. *Bacillus thuringiensis* is the only bacterium to successfully control Colorado potato beetle. Until recently, one of the drawbacks to testing bacteria against the Colorado potato beetle has been the lack of an artificial diet for screening. Previous artificial diets will only be consumed by Colorado potato beetle larvae when fresh. To improve storage, we developed a freeze-dried diet, based on a 96-well plate, suitable to feed larvae for the duration of a bioassay. Individual diet components were tested both for their effect on insect growth and on pathogen toxicity. When the preservatives, methylparaben and sorbic acid, were removed from the diet, the average weight of second instar larvae increased from 7.9 mg to greater than 9.8 mg. The preservatives inhibited the growth of two of the bacteria tested, *Photobacterium luminescens* HM and *Chromobacterium* sp. PRAA. The removal of these preservatives also allowed for fungal growth and reduced survival from 94 to 38%. Removing diet preservatives, that inhibited the growth of *Chromobacterium* sp. PRAA, increased the total mortality of the larvae as well as reducing the time needed to kill 50% of the larvae. Compared to incorporation of bacteria into molten diet, the total mortality of Colorado potato beetle fed either *P. luminescens* HM or *Chromobacterium* sp. PRAA on freeze-dried diet doubled. Preparation of freeze-dried diet need not be synchronized with the insect or the pathogen. The freeze-dried diet gave consistent results as measured by low control mortality and pathogen toxicity over time.

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1. Introduction

Bacillus thuringiensis Berliner is the only commercially successful bacterial pathogen for the Colorado potato beetles (Ferro et al., 1997). Other pathogens, such as *Serratia marcescens* Bizio or *Spiroplasma leptinotarsae* Hackett et al. while causing mortality of beetles in the lab, do not effectively control this pest in the field (Grimont and Grimont, 1978; Hackett et al., 1996).

Until recently Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) could not be reared on an artificial diet. The development of the IBL (Insect Biocontrol Laboratory) diet allows Colorado potato beetle to be reared year round without also raising plants (Gelman et al., 2001). However, this diet contains antibiotics and

preservatives that may affect the screening of pathogens. With Lepidoptera, assays using *B. thuringiensis* have to be performed without antibiotics and preservatives (formalin) to effectively measure toxicity (Reichelderfer, 1985).

Toxins that are midgut poisons, while safer than contact poisons to non-targets, must be consumed to be active. An alternative to leaf assays is an artificial diet (Reichelderfer, 1985). Because many of the components of lepidopteran diets are labile, artificial diets are usually made as needed. Incorporating pathogens into diet requires a certain amount of heat stability of both the toxin and the pathogen. Many Gram-bacteria are sensitive to heat and incorporating them into molten diet in this way would eliminate or reduce both the pathogen and heat-labile toxins that may be useful under ambient temperatures.

When assaying pathogens using diets that cannot be stored, the diet incorporation, insect instar, and

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pathogen stage must be synchronized. A pre-made diet that can be used at any time would eliminate the need for timing of making the diet. Simultaneously, a pre-made diet would also solve problems with heat-inactivated pathogens and could address the effects of antibiotics or other inhibitory compounds of the diet on individual pathogens. One of the ways that diet can be pre-made is through freeze drying (Dowd et al., 1998).

2. Materials and methods

2.1. Bacterial strains and inhibition

Photorhabdus luminescens (Poinar and Thomas) strain HM was a subculture of the type strain Hm (obtained from D. Bowen, University of Wisconsin). *Chromobacterium* sp. strain PRAA was isolated from Maryland forest soil by S. Stone (Eleanor Roosevelt High School) and identified to genus by 16S ribosomal DNA sequencing (Accugenix, Newark, DE). *Bacillus thuringiensis* subsp. *tenebrionis* NTEN was obtained from Novodor FC (Abbott Labs, Chicago, IL). *Serratia marcescens* TERM was obtained from L. Carta (Nematology Laboratory, USDA, ARS, Beltsville, MD). *B. thuringiensis* NTEN was cultured on T3 (Travers et al., 1987) and all other bacteria were cultured in L-medium (ATCC Medium 1154, Atlas, 1997) or on L-agar at 25 °C.

To test for inhibition, diet components were made up separately at the concentrations in the diet. Paper disks (6 mm, Difco, Detroit, MI) were soaked in solutions of soluble diet components or suspensions of insoluble diet components for at least 30 min and placed on a freshly spread lawn of bacteria on L-agar. Petri plates (100 mm) were incubated at 25 °C for 48 h. Clear zones, including the 6 mm disk, with no bacterial growth were then measured with digital vernier calipers. Paper disks soaked in water were also measured as the control. Each compound was tested three to five times.

2.2. Insects

The Colorado potato beetle colony, in the Insect Biocontrol Laboratory, originated from eggs sent from the New Jersey Department of Agriculture in 1996. The colony has been maintained on potato foliage. Field-collected insects from Beltsville, Maryland are introduced yearly to maintain genetic diversity. Colorado potato beetle adults were fed potato foliage and eggs laid on potato foliage were harvested, hatched, and placed on diet. Insects were reared from eggs for bioassays on IBL potato leaf diet in 100 mm × 20 mm petri dishes in paper bags. The IBL diet was a modification of the Forester diet (Gelman et al., 2001) made with defined ingredients as well as potato leaf powder, tomato fruit powder and neomycin. Per liter batch the ingredi-

ents are: 60 g torula yeast (ICN, Biomedicals, Aurora, OH), 40 g rolled oats (Quaker Old Fashioned), 30 g lactalbumin hydrolysate (Bioserve, Frenchtown, NY), 10 g casein (Bioserve, Frenchtown, NY), 25 g potato leaf powder (Superior), 12.5 g tomato fruit powder (cv. "Better Boy"), 20 g fructose (USB, Cleveland, OH), 12 g Roche vitamin mix (Bioserve, Frenchtown, NY) 4 g Beck's salt mix (Bioserve, Frenchtown, NY) 1 g β -sitosterol (USB, Cleveland, OH), 0.8 g methyl paraben (USB, Cleveland, OH), 0.8 g sorbic acid (Bioserve), 0.2 g neomycin sulfate (ICN), 2 ml wheat germ oil (ICN, Biomedicals, Aurora, OH), 2 ml soybean oil (Wesson), 14 g agar (Bioserve), and 768 ml distilled water. Incubation was initially in the dark so that the larvae would feed on the diet, and then on a light–dark regime of 16:8 (L:D) with 46% relative humidity (RH) at 24 °C. Diet was changed every 4 days.

2.3. Freeze-dried diet

For bioassays the diet was used as re-hydrated freeze-dried pellets. The standard diet was made as described without neomycin. It was poured into 96-well polystyrene plates (GreinerBioOne, Longwood, FL), frozen overnight (–20 °C), and dried in a Virtis Advantage Freeze Drier (The Virtis, Gardiner, NY) under the following conditions. Frozen diet in 96-well plates was placed on shelves that were frozen to –45 °C and held for 20 min. The diet was dried in nine steps under vacuum at 15 mTorr: –40 °C for 600 min, –30 °C for 420 min, –20 °C for 300 min, –10 °C for 300 min, 0 °C for 60 min, 10 °C for 60 min, 20 °C for 120 min, 30 °C for 120 min, and 40 °C for 120 min. The first four steps are the primary drying phase and the last six steps are needed for secondary drying. Without secondary drying, the pellets tended to be spongy and did not absorb liquid well. After releasing the vacuum, the 96-well plates were removed from shelves, dried diet pellets were removed from the 96-well plates, placed in sterile plastic bags, and stored at 4 °C before use. The dried diet pellets, which had lost 0.302 ± 0.011 g/pellet (mean \pm SEM), were stored in sterile plastic bags at 4 °C until used.

To test the effects of different diet components on growth of larvae during the assay, several different diets were made and freeze-dried as above. As a standard diet, IBL diet was made without neomycin, as used for Colorado potato beetle bioassays. Using this diet as a base, the test diets were: +neomycin as used for rearing the larvae, –methylparaben–sorbic acid, and –tomato fruit powder. Each diet pellet was placed in a well (1.6 cm diameter × 1.6 cm deep) of a white plastic bioassay tray (C–D International, Ocean City, NJ). Each type of diet was re-hydrated with 0.3 ml sterile water and one second instar Colorado potato beetle larva was added to each pellet. The larvae were weighed before placing on diet and after 120 h on diet, which is the usual

length of a bioassay. Thirty-two insects were used for each test diet.

2.4. Insect bioassays

To test the effect of heating on pathogen survival and toxicity, about 10^8 colony forming units (cfu) of each pathogen were incorporated into 125 g of molten standard diet at the final mixing and poured into 96-well plates. Concentration per pellet was calculated from the amount of pathogen added divided by the number of wells poured. Diet pellets were removed from the 96-well plate when they had solidified and were used as described below. The bacteria were added as an aqueous suspension to freeze-dried diet by volume at the same concentration as incorporated into the molten diet. Bacteria were recovered from both diets by resuspending the diet pellets in 5 ml of water, blending for 60 s on the high setting using a Stomacher 80 blender (Tekmar, Cincinnati, OH) and plating on L-agar.

For each treatment in a bioassay, 32 diet pellets were used. For freeze-dried diets, pellets were either re-hydrated with 0.3 ml, determined from the loss in weight from the fresh pellets, of water (controls) or suspensions containing dilutions of the pathogen (treatments). One second instar Colorado potato beetle larva was added to each diet pellet. Trays containing pellets were covered with bioassay tray covers (C–D International, Ocean City, NJ). Holes were made in the covers with insect pins. Insects were incubated as described for rearing and mortality was recorded at 24, 48, 72, 96, and 120 h. Cell counts were used because the specific mode of action is not yet known for all pathogens, and cell counts could be used to compare pathogens with unknown modes of action. Assays with control mortality above 5% were discarded.

Because *Chromobacterium* sp. PRAA was most inhibited by methylparaben and tomato fruit powder, bioassays were performed incorporating *Chromobacterium* sp. PRAA into the standard diet without neomycin and also to diets without methylparaben or tomato fruit powder.

2.5. Statistical analysis

LT₅₀s were also calculated from the PROBIT procedure (SAS Institute, 1999) for the hour at which the

mortality was recorded with 95% confidence intervals. Differences in weights were analyzed using MIXED procedure and means were separated using least significant difference, $\alpha = 0.05$ and a macro (Saxton, 1998).

3. Results

3.1. Inhibitory compounds

Most of the ingredients used to make up the diet were not inhibitory to the four pathogens tested including: wheat germ, rolled oats, potato leaves, and vitamins as the bacteria grew over the disks. All four pathogens tested were inhibited severely by neomycin (200 µg/g). The zones of inhibition around the disks with neomycin measured 17.07 ± 0.87 mm for *S. marcescens* TERM, 17.40 ± 0.57 mm for *B. thuringiensis* NTEN, 18.95 ± 0.62 mm for *P. luminescens* HM, and 21.93 ± 0.79 mm for *Chromobacterium* sp. PRAA. However, the preservative methylparaben (2.6 mg/g) was slightly inhibitory to only *P. luminescens* HM (6.8 ± 0.11 mm) and moderately inhibitory to *Chromobacterium* sp. PRAA (13.83 ± 0.32 mm). Only *Chromobacterium* sp. PRAA was slightly inhibited by tomato fruit powder (6.98 ± 0.2 mm). Neither *B. thuringiensis* NTEN nor *S. marcescens* TERM were inhibited by the preservatives or by any other component of the diet. Since *P. luminescens* HM and *Chromobacterium* sp. PRAA were the most sensitive of these pathogens to diet components, and they caused the greatest mortality to second instar Colorado potato beetle larvae (Martin, 2002) they were used in subsequent studies.

3.2. Effects of diets on insect growth and survival

When insect growth on various diets was compared, there was no difference in final weights of the insects. The initial larval weight was 3.28 ± 0.06 mg. The highest final weights were obtained with larvae fed on the diet that contained no neomycin or no sorbic acid (Table 1), but the survival was only 37.5%. Since differences in final weight were not significant ($F = 1.45$, $df = 4124$, $P = 0.2205$), choice of diet for further studies was based on larval survival and the presence of fungi when larvae were weighed. Fungi were present on diet pellets and on

Table 1
Comparisons of diets containing pathogen inhibitory components^a

Diet	% Survival	Initial weights (mg \pm SEM)	Final weights (mg \pm SEM)
With neomycin	96.9	3.38 ± 0.11 ab	8.74 ± 0.74 a
Without neomycin	93.8	3.48 ± 0.14 a	7.92 ± 0.80 a
Without methylparaben	90.6	3.12 ± 0.14 ab	9.84 ± 0.87 a
Without tomato fruit powder	78.1	3.34 ± 0.13 ab	7.53 ± 0.72 a
Without sorbic acid	37.5	3.05 ± 0.13 b	9.96 ± 1.58 a

^a Means within the same column followed by the same letter are not significantly different ($P < 0.05$. LSD one-way ANOVA, Saxton, 1998).

Table 2

Mortality of second instar Colorado potato beetle fed fresh or freeze-dried artificial diet containing bacterial pathogens

Strain	Diet	% Mortality	LT ₅₀ in hours (95% CL)
<i>Chromobacterium</i> sp. PRAA	Fresh	28.1	153.4 (121.8–251.6)
	Freeze-dried	56.3	100.3 (85.7–125.7)
<i>P. luminescens</i> HM	Fresh	46.8	118.8 (102.3–153.23)
	Freeze-dried	84.4	77.4 (69.1–86.0)

the larvae on diets which did not contain the preservatives sorbic acid and methylparaben when the larvae were weighed at the end of the experiment. Fungi did not grow on the diets containing the preservatives, sorbic acid and methylparaben, after 6 days. For every diet tested, one to five larvae did not gain weight.

3.3. Comparisons of pathogenicity by diet

For both *Chromobacterium* sp. PRAA and *P. luminescens* HM, the freeze-dried diet had double the mortality as compared to incorporation into molten diet (Table 2). The LT₅₀ also decreased by a third for both strains on freeze-dried diet.

When two inhibitory compounds (methylparaben and tomato fruit powder) were individually left out of the freeze-dried diet, the total mortality increased and the LT₅₀ decreased for second instar larvae treated with *Chromobacterium* sp. PRAA. For diet without tomato fruit powder the mortality increased from 34.4 to 71.9% at 120 h, but for diet without methylparaben the increase in mortality was only from 63.9 to 75%. The LT₅₀ for larvae treated with *Chromobacterium* sp. PRAA, was reduced from 132.1 h (118.1–165.5) to 96.2 h (88.2–106.0) when the tomato fruit powder was eliminated from the diet. For larvae on diet with this pathogen and without methylparaben, the LT₅₀ was reduced from 100.3 h (91.6–111.9) to 89.7 h (80.0–100.5).

Neither *Chromobacterium* sp. PRAA nor *P. luminescens* HM could initially be recovered when incorporated into molten diet when approximately 10⁶ bacteria/g diet were initially added (detection limit 80 cfu/g diet). However, 2.25 × 10⁵ cfu/g of *Chromobacterium* sp. PRAA and 7.5 × 10³ cfu/g *P. luminescens* HM were recovered initially from re-hydrated freeze-dried diet pellets. *P. luminescens* HM could be detected in diet up to 7 days (80–150 cfu/g), but viable *Chromobacterium* sp. PRAA could not be recovered from freeze-dried diet even after 1 day.

The temperature of the diet when the pathogen was added was approximately 75 °C. It took between 15 and 20 min to cool to room temperature when poured into 96-well plates.

3.4. Diet variability

In 10 separate bioassays performed over the course of 2 years on the standard diet without neomycin, the

Table 3

Variability of mortality of second instar Colorado potato beetle fed a single dose of *Chromobacterium* sp. PRAA approximating an LC₅₀ at 96 h

Batch No.	Age of diet (days)	% Mortality	LT ₅₀ in hours (95% CL)
1	8	39.4	110.3 (92.0–162.6)
1	106	46.9	98.1 (88.1–117.9)
2	31	37.5	105.5 (92.5–137.2)
2	45	37.5	107.5 (89.8–159.4)
2	69	60.0	88.1 (76.4–109.8)
2	83	63.6	82.7 (72.4–99.0)
3	1	48.9	93.0 (83.3–110.2)
4	24	53.1	91.8 (77.1–125.5)
4	35	42.9	97.2 (88.8–113.2)
4	55	37.6	102.3 (89.9–130.8)
Mean		46.7	99.8 (92.6–110.0)

variability in mortality for a concentration approximating the LC₅₀ at 96 h for *Chromobacterium* sp. PRAA ranged from 37.4 to 63.9% (Table 3). Freeze-dried diets for Colorado potato beetle have been used in more than 130 bioassays over the same two year period. The age of the diets ranged from 1 day to more than 3 months. Although there were differences in the control mortality, total pathogen mortality, or LT₅₀ based on the age of the diet, the trends were minor and could be opposite for different batches of diet (Table 3).

4. Discussion

Artificial diets can adversely affect the ability of bacterial pathogens to kill Colorado potato beetle larvae. The presence of antibiotics or inhibitory compounds substantially reduced the mortality of beetle larvae when exposed to some bacteria. The antibiotic, neomycin, which is normally included in the artificial diets used for rearing (Gelman et al., 2001), also greatly inhibited the growth of all four bacterial species tested. Studies on Lepidoptera have demonstrated that antibiotics in artificial diets reduce or eliminate the toxicity of bacterial pathogens like *B. thuringiensis* (Reichelderfer, 1985). Bacteria that do not form spores and produce toxins may also be unstable when added to molten diet.

The inhibitory effects of other diet components on the pathogens were not universal. The preservative methyl-

parben inhibited the growth of only *Chromobacterium* sp. and *P. luminescens* with no effect on the growth of either *B. thuringiensis* or *S. marcescens*. Tomato fruit powder inhibited the growth of only *Chromobacterium* sp.

While the above-mentioned compounds inhibited the growth of the pathogens, the effect on short-term insect growth and survival was not known. Eliminating sorbic acid (another preservative) from the diet, while increasing the weight of surviving Colorado potato beetle larvae, led to 62.5% mortality due to fungal contamination. Elimination of all other compounds had a non-significant effect on weight of larvae after 6 days. Weighing individual insects showed that between 1 and 5 larvae in each assay did not eat, which may explain why in bioassays for oral pathogens or toxins, the mortality does not always reach 100%. Without pathogens, larval survival was greater than 90% when they were fed diets with the full complement of ingredients or diets missing neomycin or neomycin and methyparaben. Longer term effects on fertility and fecundity were not tested. The presence of fungi on diet without methylparaben may introduce interactions with the bacterial pathogen being tested. Thus, we used a standard diet without neomycin, but including methylparaben, sorbic acid, and tomato fruit powder for further toxicity and survival studies.

How long pathogens survive on any food source for Colorado potato beetle is unknown. While *P. luminescens* HM could be recovered for 7 days when added to freeze-dried diet, incorporation into molten diet reduced the initial titer from 10^6 bacteria/g diet to below the limit of detection. *Chromobacterium* sp. PRAA did not survive well in either diet.

The decrease in recovery of bacteria incorporated into molten diet reflected decreases in mortality. Both *Chromobacterium* sp. PRAA and *P. luminescens* HM caused more larval mortality when added to freeze-dried diet than to molten diet. Because of the lack of the heat step, the addition of the pathogens to freeze-dried diet is more comparable to application of pathogens to potato leaves.

Proteins are susceptible to denaturation by heat. Therefore, it is reasonable that protein toxins such as those of *P. luminescens* (Bowen and Ensign, 1998) are less toxic to Colorado potato beetle larvae after exposure to heat in molten diet than when applied to freeze-dried diet.

That other bacteria such as *Chromobacterium* sp. PRAA are also less toxic to Colorado potato beetle larvae when added to molten diet may explain why few other Gram-pathogens have been exploited for insect control (Lacey et al., 2001).

Freeze-dried diet has additional benefits. Large batches can be made to maintain consistency. This consistency is demonstrated in that mortality in bioassays of Colorado potato beetle larvae using a dilution near the LC_{50} for *Chromobacterium* sp. PRAA varied only be-

tween 38 and 64% over a 2-year-period (Table 3). The LT_{50} also varied only from 82 to 110 h during the same period. The making of diet need not be synchronized with pathogen growth and insect stage. If a diet component inhibits a particular pathogen, a separate diet without that component can be made for short-term testing. Pathogens and larvae can be easily added to individual pellets to increase the number of replicates. The concentration of pathogen can also be readily altered. Less diet is wasted since all diet can be made into pellets and stored for future use at least 4 months. Therefore, freeze-dried diets are useful to screen and characterize pathogens for Colorado potato beetle. We have also freeze-dried the gypsy moth diet (Bell et al., 1981) and are using it to test bacterial pathogens of gypsy moth larvae.

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